



ORIGINAL ARTICLE

Fungal endophytes isolated from *Protium heptaphyllum* and *Trattinnickia rhoifolia* as antagonists of *Fusarium oxysporum*



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Abstract Control of fungal pathogens is mainly addressed by the use of chemically synthesized fungicides which result in environmental pollution, developing resistance after prolonged use. In this context, endophytes have been recognized as potential biocontrollers, and also as a promising source of antifungal metabolites. Therefore, as part of our research on phytopathogen controllers, 355 fungal endophytes were isolated from *Protium heptaphyllum* and *Trattinnickia rhoifolia* (Burseraceae), both ethnobotanically important tree species that produce secondary metabolites of agronomic and industrial interest. Endophytes were tested by *in vitro* dual culture against *Fusarium oxysporum*, a phytopathogen of agronomic importance. Five endophytes exerted at least 40% inhibition on *F. oxysporum* growth. Ethyl acetate (EtOAc) extracts were obtained from the most active antagonistic fungi, after growing them in three different liquid media. The extracts were tested against a conidial suspension of *F. oxysporum* by direct bioautography. Two extracts derived from fungi identified as *Chaetomium globosum*, F211_UMNG and *Meyerozima* sp. F281_UMNG showed inhibition of pathogen growth. Isolate *C. globosum*, F211_UMNG was selected for a chemical analysis by RP-HPLC-DAD-ESI-MS and antifungal molecules such as cladosporin, chaetoatrosin A and chaetoviridin A were annotated and identified based on their MS data.

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PALABRAS CLAVE

Endófitos;
Burseraceae;
Chaetomium;
Metabolitos

Endófitos fúngicos aislados de *Protium heptaphyllum* y *Trattinnickia rhoifolia* como antagonistas de *Fusarium oxysporum*

Resumen El control de patógenos fúngicos se basa principalmente en el uso de fungicidas de síntesis química, los que pueden dar lugar a la contaminación del medio ambiente y el desarrollo de resistencia después de un uso prolongado. En este contexto, los endófitos han sido reconocidos como potenciales biocontroladores y también como fuentes prometedoras de metabolitos secundarios antifúngicos. En el marco de nuestra investigación sobre controladores de fitopatógenos, se aislaron 355 hongos endófitos de *Protium heptaphyllum* y *Trattinnickia rhoifolia* (Burseraceae), especies arbóreas de valor etnobotánico que producen metabolitos secundarios de interés agronómico e industrial. Los endófitos fueron evaluados *in vitro* en cultivos duales frente a *Fusarium oxysporum*, un fitopatógeno de importancia agronómica. Cinco endófitos mostraron al menos un 40% de inhibición en el crecimiento de *F. oxysporum*. Una vez determinados los hongos más activos, estos se cultivaron en 3 medios líquidos diferentes y a partir de ellos se preparó una serie de extractos solubles en acetato de etilo. Los extractos fueron probados contra una suspensión de conidios de *F. oxysporum* por bioautografía directa. Dos extractos derivados de los hongos identificados como *Chaetomium globosum* (F211_UMNG) y *Meyerozima* sp. (F281_UMNG) mostraron inhibición del crecimiento del patógeno. En el extracto derivado del hongo *C. globosum* se anotaron e identificaron los compuestos antifúngicos cladosporina, chaetoatrosina A y chaetoviridina A mediante el análisis por RP-HPLC-DAD-ESI-MS. © 2017 Asociación Argentina de Microbiología. Publicado por Elsevier España, S.L.U. Este es un artículo Open Access bajo la licencia CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Using chemically synthesized fungicides has been the first line strategy to control phytopathogenic fungi³¹. However, secondary effects, such as environmental pollution and resistance development due to the use of these products, has led to a growing reluctance to use hazardous fungicides in agriculture. Thus, an enhanced trend in searching new control strategies involving environment-friendly alternatives in the management of plant pathogens has arisen²⁰. In the search for such control strategies, naturally-occurring chemical entities have become potential alternatives for the industry to replace synthetic products²³. In this context, microorganisms constitute a rich source of compounds with useful properties⁴⁸ for several applications in the agrochemical and pharmaceutical industries^{11,23}.

For several decades, the interaction between fungal endophytes and their hosts has attracted the researchers' attention, mainly because of the advantageous characteristics they confer to their host. Among these characteristics we can mention enhanced stress tolerance, plant growth factor production, herbivore repellency and protection against pathogens¹⁸. The latter characteristic is partly due to the fact that endophytes compete with other microorganisms for a specific niche, which could be achieved by the production of antibiotic-like secondary metabolites, along with other strategies⁴. As a consequence of their repellent properties, endophytes have been proposed as biocontrollers and as a promising source of antifungal metabolites against phytopathogens of agronomic importance¹⁸.

Based on our ongoing search for biologically active secondary metabolites from endophytic fungi, the objective of this work was to explore the diversity of endophytes isolated

from *Protium heptaphyllum* and *Trattinnickia rhoifolia* (Burseraceae) from Casanare, Colombia. These tree species, have been traditionally used by indigenous communities to treat several ailments¹², and their complex chemical repertory has provided useful compounds having industrial, pharmaceutical and agronomic potential^{38,43}. Furthermore, endophytes have been isolated from a species of the Burseraceae family, such as *Muscodor yucateensis*²⁵, with potential for controlling phytopathogens. Therefore, the aim of this work was to test *in vitro* the abilities of endophytes to inhibit the mycelial growth of *Fusarium oxysporum*, by metabolite production. *F. oxysporum* is a pathogen of many plant species that represent a major threat for the production of several agriculturally important crops, such as banana, carnation, chickpeas, dates, lentils, tomato, and others²⁷. The active component or components, responsible for the antifungal activity were partially characterized following a bioassay-guided fractionation test of the liquid culture-derived crude extract from the most antagonistic endophyte, to be incorporated in the future to control management programs for plant pathogen *F. oxysporum*.

Methods

Recovery of endophytes and isolation

A total of two individuals from *P. heptaphyllum* and two from *T. rhoifolia* were collected in the foothill of the west Colombian Andes mountains in Aguazul, Casanare, Colombia (N 05°13'47.89", W 072°30'31.38"), a transition ecosystem between the savanna and the high Andean ecosystems. Botanical specimens of *P. heptaphyllum* (Aubl.) Marchand (COL573961) and *T. rhoifolia* (Aubl.) Marchand (COL573962)

were deposited in the Colombian National Herbarium. From each tree, the plant material (from higher, medium and lower strata) was sampled in order to collect representative isolates from all the plants. Five leaves per level were collected in a total of 60 leaflets that were bagged in sealed bags and stored in dark conditions for 24 h at room temperature (ca. 26 °C). Petioles were then removed and the complete leaves were vigorously washed with distilled sterile water and Tween 20 (0.01%), then submerged in 70% aqueous ethanol (1 min), then in 1% sodium hypochlorite (3 min), and then rinsed three times with sterilized distilled water. Leaves were then imprinted on Potato Dextrose Agar (PDA, Oxoid, UK) for verifying the disinfection of all epiphytic microorganisms.

Each leaf was sectioned into 2 mm² pieces, and 5 randomly-chosen pieces from each leaf were seeded in Petri dishes (90 mm × 15 mm) containing water agar (agar 1.5%) (WA), 1/10 PDA (PDA at a 10th of the recommended concentration) or PDA, and then incubated at 26 °C. Hyphae tips emerging from the leaf pieces were collected for three weeks, and sub-cultured on PDA at 26 °C in the dark. Axenic cultures were established and, when a specific fungus sporulated, a monosporic culture was established. Those fungi that never sporulated were kept for a hyphal tip culture.

Identification of endophytes

Fungal populations were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores^{3,14,21}. Fungi were identified up to the genus level by observing the presence of conidial mycelium, spore mass color, distinctive reverse colony color, production of diffusible pigments, and spore morphology³. Cultures that repetitively failed to sporulate on different media were recorded as *mycelia sterilia*.

Additionally, those endophytes that inhibited *F. oxysporum* growth above 40% were identified by amplification of the nuclear ribosomal internal transcribed spacer (ITS) region, using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4(5'-TCC TCC GCT TAT TGA TAT GC-3')⁴⁶. Amplicons were sequenced with the same primers bidirectionally a single time in Macrogen Inc. (Korea), and the resulting sequences were aligned and edited in BioEdit v7.2.5¹³. The sequences were confronted with those in GeneBank database (<http://www.ncbi.nlm.nih.gov>), using BLASTN 2.2.28²⁸. The closest match was selected and aligned using ClustalW²⁶. For the phylogenetic analysis,

tree constructions were done with the MEGA 6.0 program package⁴¹ using the neighbor-joining method³⁷. Bootstrap analysis was done using 1000-times resampled data. The resulting sequences were deposited in the GenBank.

Ethyl acetate (EtOAc) extraction

Fungi selected for their inhibitory activity at *in vitro* conditions against *F. oxysporum* were reactivated in 500 ml of Potato Dextrose Broth (PDB, Oxoid, UK), Sabouraud Broth (SAB, Oxoid, UK) and yeast extract sucrose media (YES)¹⁹, and cultured in an orbital shaker under constant agitation (100 rpm) at 21 °C for 7 days. After that period the culture was filtered using Whatman No. 1 qualitative filter paper, and mycelia were lyophilized. Separately, both mycelia and filtered media were mixed with EtOAc in a 1:3 proportion and incubated in an orbital shaker in constant agitation (100 rpm) for 48 h. The organic phase (EtOAc) was separated from the mycelia by vacuum filtration using Whatman No. 1 qualitative filter paper, and from filtered liquid media using a decantation funnel. The resulting extracts were concentrated by lyophilization.

Antifungal assays

Fungal endophytes and a phytopathogenic isolate (*F. oxysporum* G1 isolated from *Physalis peruviana* (Cape gooseberry) available in the collection of the phytopathology laboratory at Universidad Militar Nueva Granada) were cultured on PDA at 26 °C for 5 days at 26 °C in the dark. In order to evaluate the possible effect of each endophyte on phytopathogen growth, dual cultures were settled and each isolate was challenged with *F. oxysporum* G1. Thus, a plug (3 mm diameter), which was obtained from the colonial actively growing edge of the endophyte to be tested, was seeded on PDA, 10 mm away from the edge of a Petri dish (90 mm × 10 mm). At a spot distance (10 mm) from the diametrically-opposed edge, a similar plug of *F. oxysporum* was seeded. Six days later, the effect of each endophyte on *F. oxysporum* growth was observed and *F. oxysporum* colony radial measurement and distance between colonies were recorded. As control, a plug of each organism was cultured alone. These experiments were replicated three times. Results were compared by the Tukey's HSD (honest significant difference) test.

The antifungal activity of the extracts was tested by direct TLC bioautographic detection⁹. Extracts and fractions from the selected endophytes (Table 1) were diluted in

Table 1 Isolation media and closest match in phylogenetic analysis by the neighbor joining method from the most active endophytes against *F. oxysporum*

Code	Endophyte isolation conditions			Closest match	NCBI accession
	Plant	Level	Recovered from		
92	<i>T. rhoifolia</i>	Low	PDA 1/10	<i>Talaromyces amestolkiae</i>	KU184613
211	<i>P. heptaphyllum</i>	Low	WA	<i>Chaetomium globosum</i>	KU184610
219	<i>P. heptaphyllum</i>	Low	PDA 1/10	<i>Phyllosticta</i> sp.	KU184614
281	<i>P. heptaphyllum</i>	High	WA	<i>Meyerozyma</i> sp.	KU184611
299	<i>P. heptaphyllum</i>	Low	WA	<i>Xylaria grammica</i>	KU184612

Species were defined when the node was supported with ≤90 (Fig. 1).

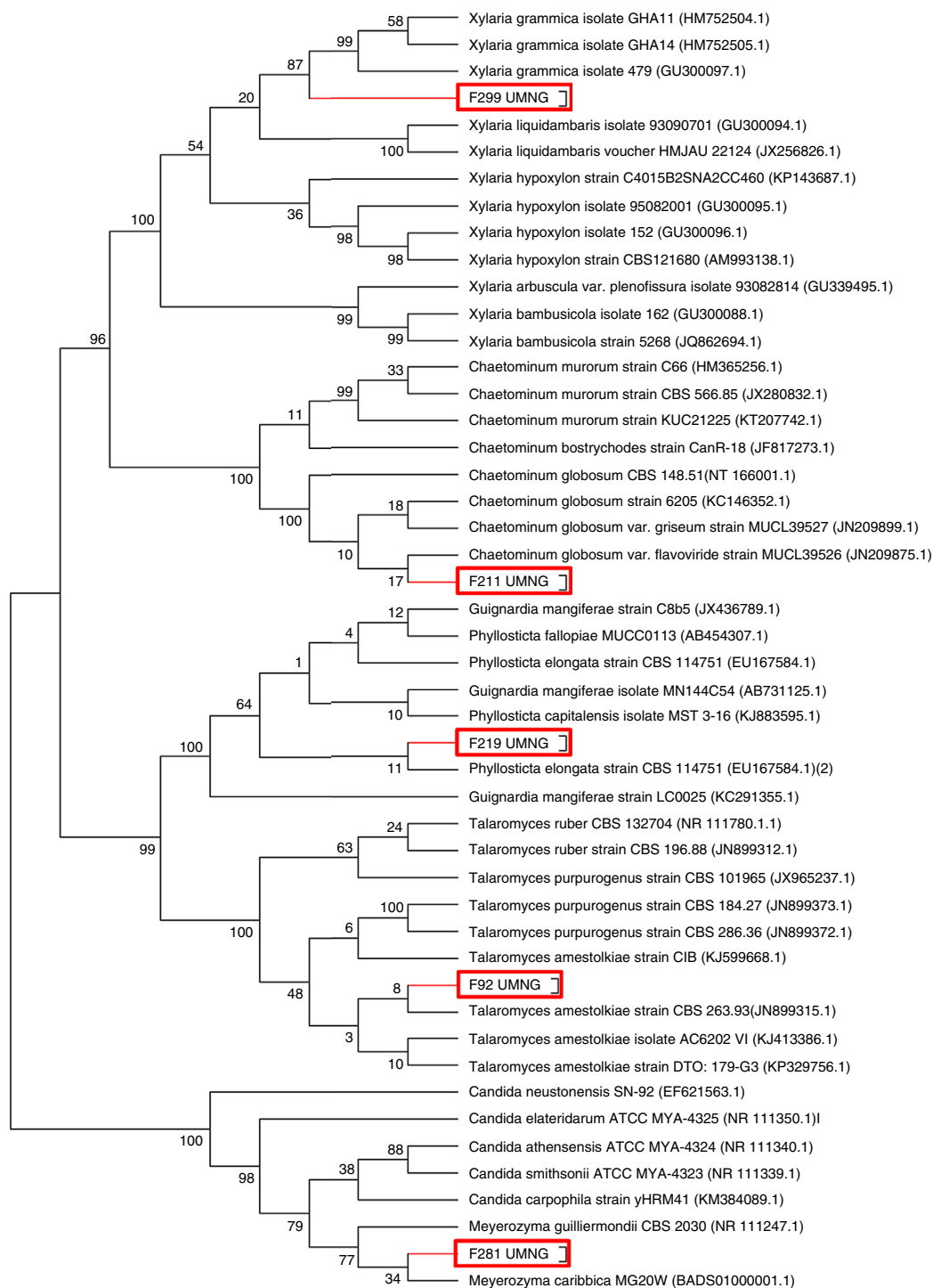


Figure 1 Dendrogram showing the phylogenetic relationship of fungal endophytes based on the ITS region. Phylogenies were inferred using the neighbor-joining analysis and trees generated in MEGA 6.0 software. Numbers at branch points indicate bootstrap values. The scale bars represent the estimated difference in nucleotide sequence. Red rectangles indicate the endophytes isolated in this work.

ethanol (HPLC grade) and 30 μg were seeded in a single spot on a TLC Aluminum silica gel 60 Sheet 20 cm \times 20 cm (Sigma-Aldrich). Then the silica sheet was sprayed with a 1×10^6 conidia/ml conidial suspension of *F. oxysporum* until the whole sheet was covered. The assays were incubated in a humid chamber for 3 days, and then *F. oxysporum* growth over the sheet was evaluated under UV-light.

Fractionation of the most active extract

The most active extract was fractionated by preparative HPLC (Shimadzu prominence LC20AD), in gradient elution, using a Shimadzu Premier column C-18 (4.6 mm \times 150 mm, 5 μm) at a flow rate of 2 ml/min. The injection volume was 50 μl . The mobile phases consisted in methanol (HPLC grade)

(Phase A) and trifluoroacetic acid 0.005% (HPLC grade) (Phase B). Separation was carried out for 25 min, in a FRC 10A Shimadzu fraction collector. A diode array detector (DAD) performed signal detection at 270 nm. A total of 20 fractions were recovered and then concentrated by lyophilization.

LC-MS-based chemical analysis

Extracts and fractions were characterized by Reverse Phase Liquid Chromatography with multi-wavelength UV-VIS detection (by a DAD) and coupled by electrospray to a mass spectrometry detector (RP-HPLC-DAD-ESI-MS) (Shimadzu Prominence LC/MS 8030). Analyses were performed on a Shimadzu prominence instrument, in gradient elution, using a Shimadzu Premier column C-18 (4.6 mm × 150 mm, 5 μm). Simultaneous monitoring was carried out at 270 nm, at a flow rate of 0.6 ml/min. The operating temperature was 30 °C and the injection volume was 20 μl. As mobile phase A 1% formic acid in distilled water (HPLC grade) was used, and acetonitrile (ACN) (HPLC grade) as mobile phase B; separation was performed for 33 min. The mass spectrometry detector (MSD) consisted of an electrospray ionization (ESI) source and a triple quadrupole analyzer. The mass spectrometry method consisted of a scan in simultaneous positive and negative ionization with an acquisition time of 2–33 min, a mass range of 50–2000 *m/z*, a scan speed of 1667 μ/s, an event time of 0.5 s, nebulizer gas flow of 1.5 l/min, 350 °C interface temperature and DL, and 450 °C block temperature. The drying gas flow rate was 9 l/s. The analysis was monitored at wavelengths between 270 and 330 nm. Annotation and identification of the major and minor metabolites in the extract was performed by mass spectrometry-based analysis, complemented with the analysis of reported metabolites.

Results

Recovery of endophytes

A total of 577 endophytes were isolated from 900 cultured pieces of leaflets. A subtotal of 355 endophytes were

selected after the elimination of redundant morphotypes derived from the same leaflet. The highest number of endophytes (*n* = 236) was recovered from the lowest collection level for both species. The ITS region of the isolates was amplified and sequenced to determine the phylogenetic relationships among them (Fig. 1). A phylogenetic tree was constructed based on a 570 bp sequences and isolates clustered as follows: endophyte F92.UMNG clustered with *Talaromyces amestolkiae*, F18.UMNG with *Phyllosticta* sp., F211.UMNG with *Chaetomium globosum*, F299.UMNG with *Xylaria grammica*, and F281.UMNG with *Meyerozyma* sp. (Table 1).

Antifungal test

The antifungal ability of 355 fungal endophytes against *F. oxysporum* G1 was evaluated by the dual culture method. Five endophytes reduced the area of *F. oxysporum* growth by at least 40%, without colony contact (Fig. 2), which were grouped in a single group by the Tukey's HSD test.

EtOAc-soluble extracts obtained from two isolates, F211.UMNG and F281.UMNG, cultured in YES, exerted inhibitory effect on *F. oxysporum* by direct bioautography (Fig. 3A). On comparing the inhibitory effect produced by these two isolates on *F. oxysporum* radial growth, it was found that isolate F211.UMNG (*C. globosum*) exerted a 64% *in vitro* inhibition of *F. oxysporum* colony growth arresting its growth producing with a distance between colonies of 12.5 ± 0.6 mm while F281.UMNG caused 45% inhibition showing 5.7 ± 0.9 mm between colonies (Figs. 2 and 3A).

Since the extract from F211.UMNG isolate showed greater inhibitory activity against *F. oxysporum*, it was fractionated in order to determine the most active fractions. A total of 20 fractions were recovered (Fig. 4A) and were additionally tested by bioautography against a conidial suspension of *F. oxysporum*. Only fraction #14 (30 μg) exerted inhibition of the fungus (Fig. 3B). Fraction #14 was analyzed by LC-MS, rendering a chromatogram that included two defined signals between min 12 and min 17 (peaks 1 and 3, Fig. 4B).

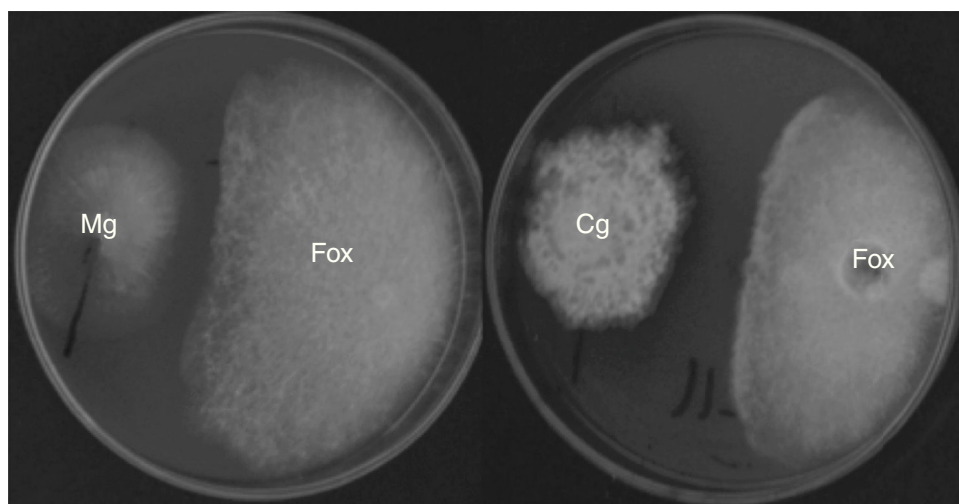


Figure 2 Inhibition of *F. oxysporum* G1 (Fox) caused by *C. globosum* F211.UMNG (Cg) and *Meyerozyma* sp. (Mg) in PDA media in dual cultures at 6 days post inoculation.

Discussion

From the recovered isolates, fungi like *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Epicoecum* sp., *Fusarium* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Xylaria* sp., among others, were identified by their morphological traits and have been previously reported as common endophytes in other plants^{6–8,22,24,29,47,49}. A high diversity of fungal species were also found in leaves and stems of *Boswellia sacra* (Burseraceae), being *Alternaria* and *Aspergillus* the most dominant genera, which were both also isolated in this work. However, *Chaetomium* was also found in a relative high proportion (26.3%) represented by two species, *C. globosum* and *C. spirale*¹⁰. Screening works in *Boswellia serrata* exhibited *Myrothecium verrucaria* and *Phoma* sp.⁴⁰ as dominant endophytes, which were also isolated in our samples.

The 355 endophytes isolated in this work were evaluated by the dual culture method against *F. oxysporum* and only five endophytes showed inhibition against *F. oxysporum* presumably by metabolite production because they inhibited the extension on the colony without mycelial contact and reduced the area of the phytopathogen by at least 40% (Table 1, Fig. 2). Antagonistic endophytes were identified by amplification of a 570 bp (ITS) region as *C. globosum*, *Meyerozyma* spp., *Phyllosticta* spp., *T. amestolkiae*, and *X. grammis*; such species have been reported as being endophytes and having antibiotic activity^{1,2,17,33,35,39,49}.

The extract from isolate *C. globosum* F211_UMNG, at 30 µg, caused inhibit they inhibited the extension on the colony ion of *F. oxysporum* growth (Fig. 3A). Based on the available literature, in the *Chaetomium* genus, mostly in *C. globosum*, seven signals defined by mass spectrum analysis

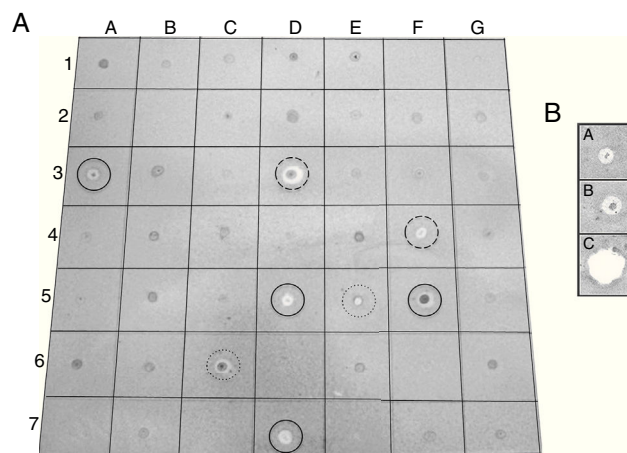


Figure 3 (A) Direct bioautography of endophyte-derived EtOAc extracts against *F. oxysporum* conidia. Solid lines: Procloraz 40 ng (control). Medium dashed line: Supernatant medium of EtOAc extract from *C. globosum* F211_UMNG. Highly dashed line: Supernatant medium of EtOAc extract from *M. guilliermondii* F281_UMNG. (B) Bioautography of F211_UMNG extract and most active fraction against *F. oxysporum* conidia. A: Fraction #14, B: Extract 211 INI, C: Sportak 40 ng.

were found to have the same *m/z* value to that reported (Table 2). Nevertheless, five isomers previously reported in the *Chaetomium* genus matched the *m/z* value detected at 38 min (peak 5, Fig. 4B) and, therefore they cannot be differentiated in accordance with the known MS limitations. Previous studies found that *C. globosum* synthesized

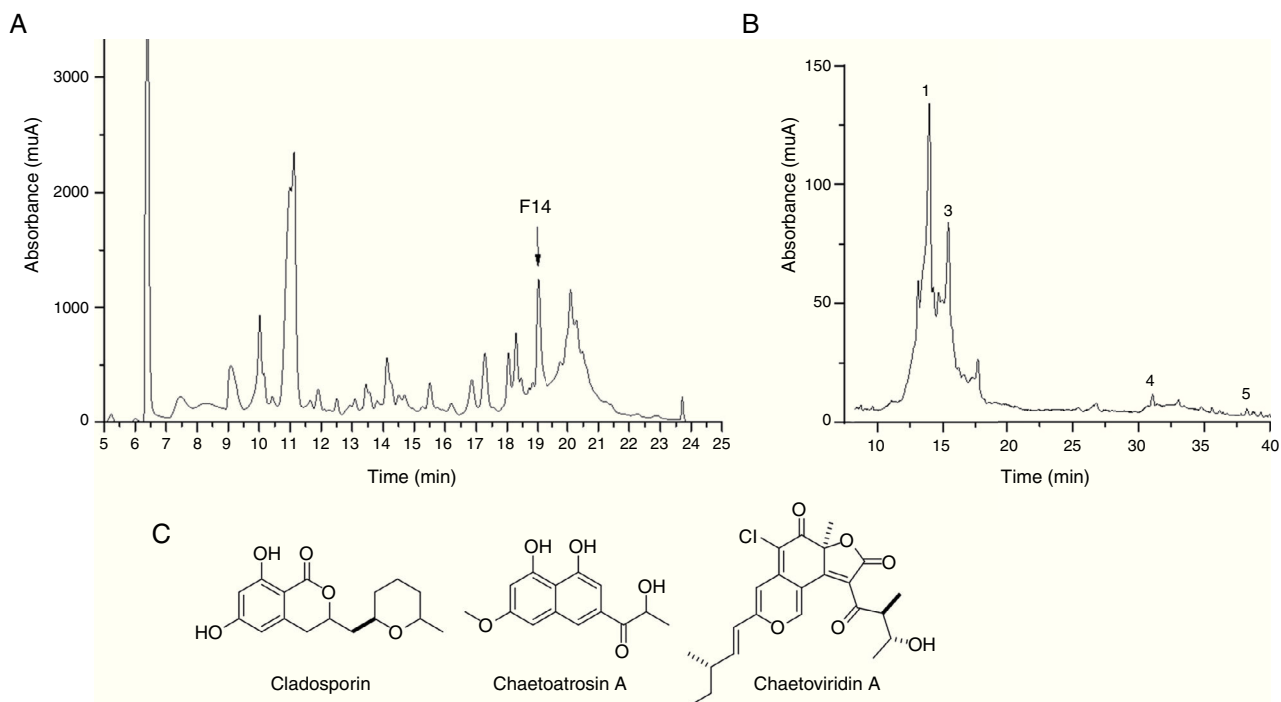


Figure 4 (A) Chromatographic profile of EtOAc extract from F211_UMNG in YES medium. F14+ arrow indicates fraction #14. (B) RP-HPLC-DAD chromatogram of fraction #14. Numbers indicate the annotated compounds by MS data (Table 2). (C) Structures of the identified compounds in the most active fraction from EtOAc-soluble extract of *C. globosum* F 211_UMNG.

Table 2 Identified compounds isolated from other *Chaetomium* strains as constituents of fraction #14

Peak ^a	t _R (min)	Compound	Isolated from	MW
1	13.0	Cladosporin ⁴²	<i>C. globosum</i> CCTCC AF 206003	292.1
2	14.6	Unknown	–	–
3	15.5	Chaetopyranin ⁴⁴	<i>C. globosum</i>	339.2
4	31.0	Chaetoatrosin A ¹⁶	<i>C. atrobrunneum</i> F449	262.1
5	38.0	Chaetomugilin C ³⁰	<i>C. globosum</i>	433.7
		Chaetomugilin N ³⁰	<i>C. globosum</i>	433.9
		<i>epi</i> -chaetoviridin A ⁵	<i>C. globosum</i>	433.9
		Chaetoviridin A ⁵	<i>C. globosum</i>	433.9
6	38.4	4'- <i>epi</i> -chaetoviridin A ⁵	<i>C. globosum</i>	433.9
		Differanisole/differanisole A ³²	<i>C. spp.</i> strain RB-001	278.0
7	39.7	Unidentified (Reaxys RN 19364999) ³⁶	<i>C. globosum</i> ZY-22	460.7

^a Peak number identifies the signals in Fig. 4B.

several molecules such as chaetoglobosins, epipolythiodioxopiperazines, azaphilones, xanthonones, anthraquinones, chromones, depsidones, terpenoids, and steroids, among others. These types of compounds have shown antitumor, cytotoxic, antimalarial, enzyme inhibitory, antibiotic, and other activities⁵⁰. In the present study cladosporin, chaetoatrosin A and chaetoviridin A (Fig. 4C) were identified to be active against *F. oxysporum*, in the EtOAc extract of *C. globosum* F211_UMNG. These compounds were previously reported as having antifungal activity^{16,34,45}. Chaetoatrosin A acts as an inhibitor of chitin synthase II, while chaetoviridin A inhibits the cholesteryl ester transfer protein (CETP)⁴². The action of cladosporin is not fully understood but it has been reported that it exhibited lysyl-tRNA synthetase inhibition in *P. falciparum*¹⁵ and that its mode of action is different to that affecting β -tubulin assembly in mitosis⁴⁵. The combination of the modes of action of the identified molecules might rationalize the observed growth inhibition of *F. oxysporum* in the *in vitro* and bioautography test.

In conclusion, five endophytes acting as antagonists of *F. oxysporum* under *in vitro* conditions were isolated and identified in the present study. Isolate *C. globosum* F211_UMNG-derived extract inhibits the growth of *F. oxysporum*, possibly by at least three molecules having different modes of action, implying its possible application in control schemes of *F. oxysporum*²⁷. A confirmation of the results through *in vivo* testing is required, involving endophyte *C. globosum* and purifying the identified compounds for evaluating their ability in the control of the disease caused by *F. oxysporum*.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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